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ENHANCED REDUCTION OF T4D AND T7 COLIPHAGE TITERS FROM *BIOMPHALARIA GLABRATA* (MOLLUSCA) HEMOLYMPH INDUCED BY PREVIOUS HOMOLOGOUS CHALLENGE

THOMAS C. CHENG, KEITH H. HOWLAND, AND JOHN T. SULLIVAN

Marine Biomedical Research Program and Department of Anatomy (Cell Biology), Medical University of South Carolina, Charleston, SC 29412

ABSTRACT

The hemolymph of *Biomphalaria glabrata* contains a soluble factor(s) that neutralizes the coliphages T4D and T7 *in vitro*. This neutralization is not enhanced by prior injection of the snail with the homologous or heterologous phage. *In vivo* titers of injected T4D and T7 decrease as a function of time postinjection (PI), with a nearly 1000-fold reduction occurring by 144 h PI. When snails are reinjected at 96 h PI with homologous phage, the *in vivo* titers of infective T4D and T7 decrease even more rapidly. Specifically, T7 titers at 24, 48, and 72 h post-reinjection (PR) are significantly lower than those at the same time periods PI, *i.e.*, following a single injection. Similarly, T4D titers at 24, 48, 72, and 96 h PR are significantly lower than at 24, 48, 72, and 96 h PI. It is concluded that the reduction of phage titer in the hemolymph of injected *B. glabrata* is due in part to neutralization by some naturally occurring factor(s) and also to an active, probably cellular, mechanism that is enhanced by previous homologous challenge. The specificity of this enhancement is not yet known; however, prior sham or Tris diluent injections do not affect the titers of a subsequent T4D or T7 injection.

INTRODUCTION

Among vertebrates, specificity and anamnesis (memory) are two primary features of the humoral and cellular immune responses. It is now generally accepted that anamnesis reflects a specific secondary response that is both more rapid and of greater magnitude than the primary response to antigenic challenge. Invertebrates, although capable of discriminating between self and nonself (see Chorney and Cheng, 1980; Hildemann *et al.*, 1980b; Lackie, 1980; and Warr, 1981 for reviews), do not possess vertebrate-type immunoglobulins. Thus, inducible memory would most likely be revealed as selectively altered reactivity upon secondary contact in some form of cell-mediated immunity (Hildemann *et al.*, 1980b). Recent data on allograft rejection in invertebrates (see Hildemann *et al.*, 1980b for review) suggests that at least in the sponges, coelenterates, echinoderms, and possibly annelids, invertebrates are capable of a specific immune response with at least short-term memory.

Although there is some evidence that bivalve molluscs, specifically oysters (*Crassostrea virginica*), can be induced to engage in enhanced clearance of T2 coliphage resulting from second challenge (Acton and Evans, 1968), the experimental protocol employed, involving half-shell preparations, is open to question. Consequently, reported herein are the results of studies on the reduction of circulating, infective coliphages from the hemolymph of *Biomphalaria glabrata*, a gastropod mollusc, which did not require removal of an entire valve. Evidence is presented that these

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molluscs possess (1) some naturally occurring phage-neutralizing factor(s) in their serum; and (2) a second, possibly cellular, mechanism that is enhanced by prior homologous challenge.

Materials and Methods

Snails

The specimens of *B. glabrata* used in this study were of the so-called NIH albino strain (Newton, 1955). All of the snails measured from 7–15 mm in shell diameter. They were maintained in 3-liter glass aquaria containing deionized water fortified with Nolan and Carriker's (1946) salt solution at a concentration of 1.58 ml/liter and fed romaine lettuce *ad libitum*.

Bacteriophages

The bacteriophages employed were the coliphages T4D and T7. They were originally obtained from Dr. L. Chao of the Department of Biochemistry, Medical University of South Carolina. The phages were harvested from *Escherichia coli* strain B (ATCC 11303). The titers of the phage suspensions used were determined weekly by the standard plaque assay method (Douglas, 1975). For injection experiments, titers of T4D and T7 were adjusted with sterile Tris diluent (1 ml of 1 *M* $MgCl_2$, 21.5 ml of 4 *M* NaCl, 10 ml of 1 *M* Tris-HCl, pH 7.5, 1 ml of 1% gelatin, and 4 ml of 0.025 *M* L-tryptophan/liter of water) to provide a suspension containing 2×10^8 plaque forming units (PFUs)/ml.

Injection procedure

The injection of snails, except for those sham injected, was accomplished by a modification of the method of Sullivan and Cheng (1976). In brief, a small aperture was made on the left side of the shell overlaying the anterior region of the digestive gland where the postintestine and midintestine lie parallel to each other. A microcapillary tube, which had been drawn to a fine point, containing 2 μl of either the phage suspension or the control solution, was inserted through the aperture into the sinus situated immediately anterior to the digestive gland. The solutions were introduced slowly as a result of pressure from a mercury column. After the microcapillary was retracted, each snail was permitted to air dry for 5 min prior to being placed in a 400-ml beaker containing deionized water fortified with Nolan and Carriker's (1946) solution. A maximum of six snails was placed in each beaker, the water was changed daily, and the animals were fed lettuce *ad libitum*.

In vitro *neutralization assay*

The ability of snail serum to neutralize bacteriophage was assayed. Fifty snails were divided into five equal groups: (1) The first group was left untampered. (2) The second group received an injection of 2 μl of the T4D suspension (2×10^8 PFUs/ml). (3) The third group received an injection of 2 μl of the T7 suspension (2×10^8 PFUs/ml). (4) The fourth group received an injection of 2 μl of sterile Tris diluent. (5) Finally, the fifth group received a sham injection (*i.e.*, inserting the needle into the hemocoel for 30 s). Snails were bled 24 h post-challenge by making a small hole in the shell directly over the heart and inserting a sterile capillary tube. At least 30 μl of hemolymph was collected from each snail. The samples were subsequently placed into individual 400-μl microcentrifuge tubes and the cells were

separated from sera by centrifugation at 1000 g for 10 min in a Beckman Model J2-21 refrigerated centrifuge. The sera were transferred to new 400-μl microcentrifuge tubes and kept at 4°C until assayed.

A 10 μl sample of both undiluted and diluted (1:100 in Tris diluent) serum from each snail from the five groups was combined with either 10 μl of a suspension of 2×10^4 PFUs of T4D phage/ml of Tris diluent, 10 μl of a suspension of 2×10^4 PFUs of T7 phage/ml of Tris diluent, or 10 μl of Tris diluent and incubated for 2 h at 25°C. Following incubation, the samples were assayed for bacteriophage by a modification of the standard plaque assay method (Douglas, 1975). Specifically, the reaction mixture, 20 μl in total, was used instead of 100 μl of serum. The plates were incubated at 25°C and the plaques were counted and recorded as PFUs/ml of serum. The assay was repeated once using a second group of 50 snails.

In vivo *assay*

The 664 snails employed in each experiment were divided into nine groups: (1) The 80 snails comprising the first group were each injected with 2 μl of the T4D suspension (2×10^8 PFUs/ml). (2) The 80 snails comprising the second group were each injected with 2 μl of the T7 suspension (2×10^8 PFUs/ml). (3) Each of the members of the third group, consisting of 80 snails, received two injections, each consisting of 2 μl of the T4D suspension, 96 h apart. (4) Each of the members of the fourth group, consisting of 80 snails, received two injections, each consisting of 2 μl of the T7 suspension, 96 h apart. (5) The 80 snails comprising the fifth group each received two injections 96 h apart; the first injection consisted of 2 μl of Tris diluent and the second injection consisted of 2 μl of the T4D suspension. (6) The 80 snails comprising the sixth group each also received two injections 96 h apart; the first injection consisted of 2 μl of Tris diluent while the second injection consisted of 2 μl of the T7 suspension. (7) Each of the 80 snails comprising the seventh group was initially sham injected followed 96 h later by an injection of 2 μl of the T4D suspension. (8) Each of the 80 snails comprising the eighth group was similarly sham injected 96 h prior to receiving an injection of 2 μl of the T7 suspension. (9) Finally, each of the 24 snails comprising the ninth group was left untampered. This injection experiment was replicated once, using an additional 664 snails.

The time interval of 96 h between injections in Groups 3 through 8 was chosen based upon the results of the single injections. The levels of viable phage remaining in the hemolymph of snails given a single injection of either T4D or T7 were relatively low at 96 h PI (approximately 1000 PFUs/ml).

Collection of hemolymph

At 6, 12, 24, 48, 72, 96, 120, and 144 h after either a single injection of coliphage or after the second injection of the paired injection series, individual hemolymph samples were collected from each of 10 snails from Groups 1 through 8 as described above. The cells were separated from sera as described above and the sera were stored for no more than 12 h at 4°C before being assayed.

Plaque assays

The serum samples were assayed from bacteriophage by employing a modification of the standard plaque assay method (Douglas, 1975). Specifically, we had to use ten times less serum (*i.e.*, 10 μl) because of the small amount of hemolymph obtainable from each snail. The plates were incubated at 25°C and the plaques were

counted and recorded as PFUs/ml of serum. If confluent plaques occurred, a serial ten-fold dilution series was prepared and assayed for bacteriophage.

Statistical analysis

The Student's two-tailed *t* test was employed to determine whether there were statistically significant differences between the mean serum titers in the control groups (Groups 1, 2, 5, 6, 7, 8, and 9) and experimentally treated groups (Groups 3 and 4) of snails in the *in vivo* assays and between the mean serum titers in the control group (untampered) and experimentally treated groups of snails in the *in vitro* neutralization assay.

Statistical analyses were performed on untransformed data. However, the data are presented graphically as the logarithm of the actual mean titers due to the wide range of titers encountered over the course of the experiments.

RESULTS

The *in vitro* and *in vivo* experiments, as explained, were repeated once, using a second group of snails for each. Since the results of all replicates were not significantly different, data were pooled, and for purposes of computing mean titer values were considered as a single large experiment.

Neutralization test

Table I presents the combined mean titers obtained from two replicate experiments in which sera from the five groups of snails were incubated with either 2×10^4 PFUs of T4D or T7/ml of Tris diluent *in vitro* for 2 h at 25°C. Statistically significant differences were observed in the following instances. Specifically, sera from snails previously challenged with either T7 or with Tris diluent and then incubated with T4D *in vitro* had a significantly higher ($P < 0.05$) mean titer than did serum from untampered snails incubated with T4D. Also, sera from snails previously challenged with either T4D or with Tris diluent and then incubated with T7 *in vitro* had a significantly higher ($P < 0.05$) mean titer than did serum from

TABLE I

Pooled results of two neutralization assays of serum from Biomphalaria glabrata *combined with the bacteriophages T4D and T7* in vitro

Serum sample	Combined with T4D (2×10^4 PFUs/ml) (n = 20 snails)	Combined with T7 (2×10^4 PFUs/ml) (n = 20 snails)
Untampered	2,480.0 ± 561.577	2,655.0 ± 551.052
T4D Injected	7,480.0 ± 8,126.150	9,280.0 ± 4,878,373[B]
T7 Injected	4,185.0 ± 1,729.093[A]	2,550.0 ± 1,417.373[D]
Tris Diluent Injected	3,570.0 ± 564.847[A,C]	3,395.0 ± 955.579[B,D]
Sham Injected	2,570.0 ± 863.347[C]	2,975.0 ± 1,096.826[D]

Entries represent the combined mean numbers of PFUs/ml of serum ± the standard deviations.
[A] Significantly greater ($P < 0.05$) than untampered snail serum combined with T4D.
[B] Significantly greater ($P < 0.05$) than untampered snail serum combined with T7.
[C] Significantly less ($P < 0.05$) than T4D injected snail serum combined with T4D.
[D] Significantly less ($P < 0.05$) than T4D injected snail serum combined with T7.

untampered snails incubated with T7. In addition, the mean titer from snails challenged previously with T4D and incubated with T4D *in vitro* was significantly higher ($P < 0.05$) than that obtained for sera from snails previously challenged with either Tris diluent or with sham injections and incubated with T4D *in vitro*. Furthermore, the mean titer from snails previously challenged with T4D and incubated with T7 *in vitro* was significantly higher ($P < 0.05$) than that obtained for sera from snails previously challenged with T7, with Tris diluent, and with sham injections and incubated with T7. No other significant differences were observed among the experimental groups.

Incubation of each serum sample from the five groups of snails with Tris diluent alone revealed that there were no phage present in the sera of any of the noninjected, the Tris diluent injected, and the sham injected snails. There were some phage remaining in the sera of all of the T4D injected and T7 injected snails. This background value for each snail was subtracted from the value obtained upon incubation of its serum with either T4D or T7 *in vitro* to obtain the mean titers shown in Table I.

Dilution of serum (1:100) with Tris diluent caused a loss in the ability of the serum to neutralize the phages. When diluted serum was incubated with T4D or T7 *in vitro* and then was assayed for phage, confluent plaques were obtained for all snails in all five groups.

In vivo *assay*

Figure 1 presents the combined mean serum titers from replicate experiments expressed as the $\log_{10}$ of the mean numbers of PFUs/ml of serum, as a function of either time after a single injection or time after the second of two injections, ascertained when *B. glabrata* were challenged with (1) a single injection of coliphage T4D, (2) a double injection of T4D administered 96 h apart, (3) an injection of sterile Tris diluent followed 96 h later by an injection of T4D, and (4) a sham injection followed 96 h later by an injection of T4D.

Statistical analyses revealed that at 6 and 12 h after the second of two injections of T4D, the mean serum titers were not significantly different from those obtained for singly injected snails or those obtained for snails that had been challenged with either an initial injection of Tris diluent or a sham injection followed 96 h later by injection of T4D. At 24, 48, 72, and 96 h after injection with the second of the two challenges with T4D, the mean serum titers for these snails were significantly less ($P < 0.05$) than those obtained from singly injected snails, snails that had been injected with Tris diluent followed 96 h later by an injection of T4D, and snails receiving a sham injection followed 96 h later by an injection of T4D. At 120 and 144 h after the second of two injections of T4D, there were no significant differences between the mean serum titers.

Figure 2 presents similar data obtained from snails subjected to the same series of injections as above, except that coliphage T7 was substituted for T4D.

Statistical analyses revealed that at 6 and 12 h after the second of two injections of T7, the mean serum titers were not significantly different from those obtained for singly injected snails and those obtained for snails that had been challenged with either Tris diluent or a sham injection initially followed 96 h later by an injection of T7. At 24, 48, and 72 h after injection with the second of the two challenges with T7, the mean serum titers for these snails were significantly less ($P < 0.05$) than those obtained for singly injected snails, snails that had been injected with Tris diluent followed 96 h later by an injection of T7, and snails receiving a sham

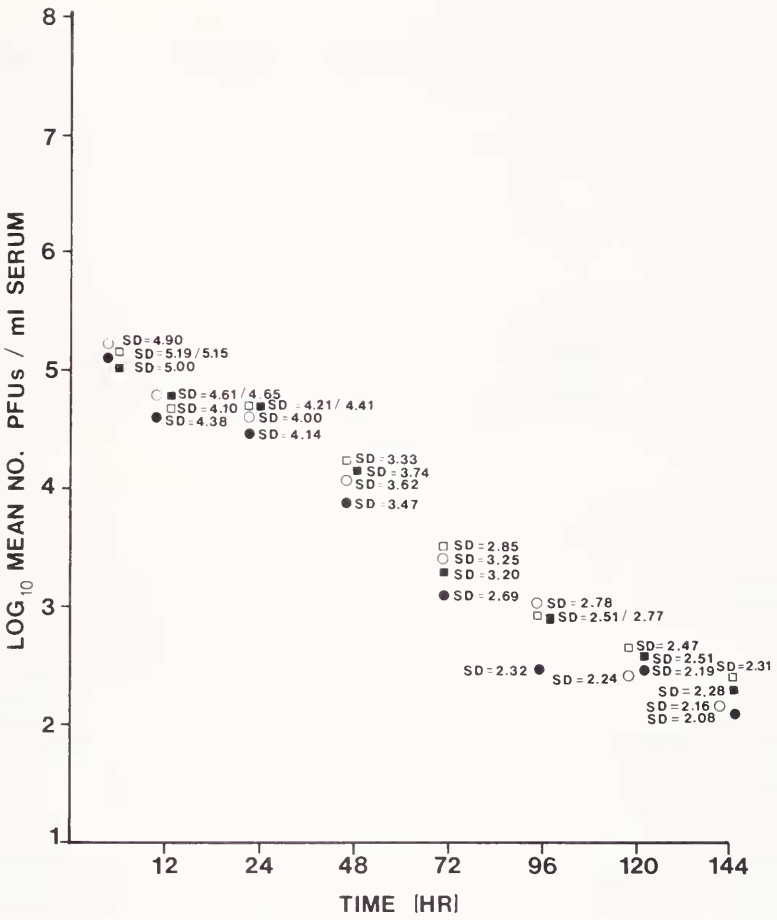

FIGURE 1. Logarithm$_{10}$ of the combined mean number of PFUs of T4D phage/ml of serum obtained from *Biomphalaria glabrata* given a single injection of T4D (○), a pair of injections of T4D (●) given 96 h apart, an injection of Tris diluent followed 96 h later by an injection of T4D (□), and a sham injection followed 96 h later by an injection of T4D (■). Each point represents the logarithm$_{10}$ of the mean number of PFUs of T4D/ml of serum from 18–20 snails, pooled from the two separate experiments. Standard deviations (SD) shown equal the logarithm$_{10}$ of the standard deviations.

injection followed 96 h later by an injection of T7. Furthermore, at 96 h after the second of two injections of T7, the mean serum titer was significantly lower ($P < 0.05$) than that for snails receiving a sham injection followed 96 h later by an injection of T7, but it was not significantly different from those for singly injected snails and snails initially challenged with Tris diluent followed 96 h later by an injection of T7. At 120 and 144 h post challenge with the second injection of T7, there were no significant differences observed between the mean serum titers.

Some mortality occurred among eight of the nine groups of snails. Specifically, the mortality rates among members of Groups 1 through 8 were as follows: Group 1, 5 out of 160 (3.1%); Group 2, 4 out of 160 (2.5%); Group 3, 3 out of 160 (1.9%); Group 4, 4 out of 160 (2.5%); Group 5, 4 out of 160 (2.5%); Group 6, 9 out of 160 (5.6%); Group 7, 4 out of 160 (2.5%); and Group 8, 4 out of 160 (2.5%).

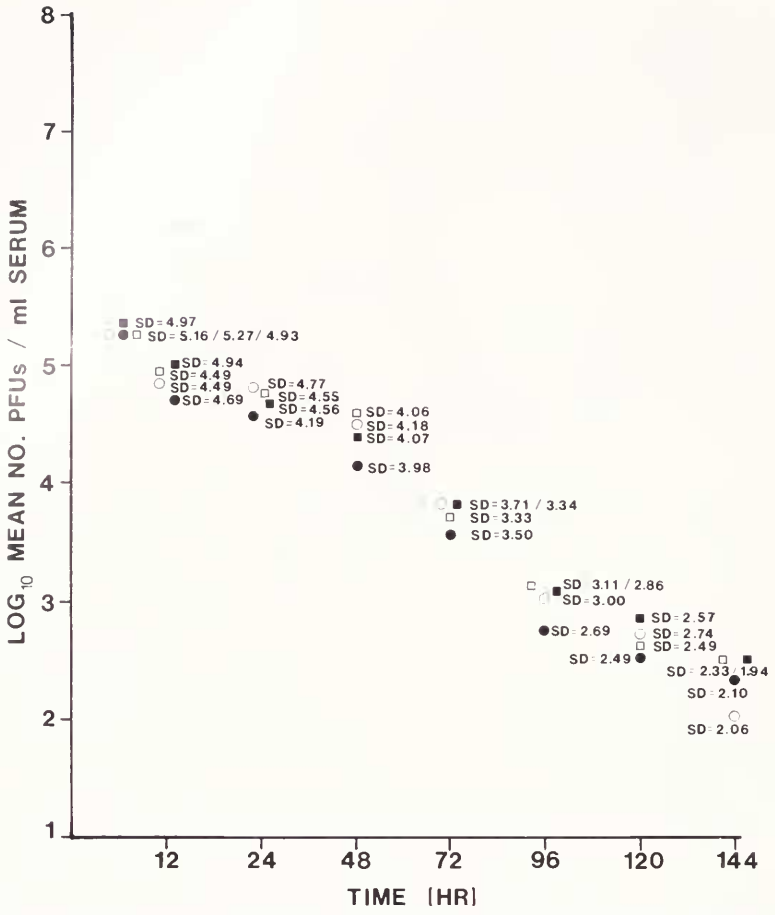

FIGURE 2. Logarithm$_{10}$ of the combined mean number of PFUs of T7 phage/ml of serum obtained from *Biomphalaria glabrata* given a single injection of T7 (○), a pair of injections of T7 (●) given 96 h apart, an injection of Tris diluent followed 96 h later by an injection of T7 (□), and a sham injection followed 96 h later by an injection of T7 (■). Each point represents the logarithm$_{10}$ of the mean number of PFUs of T7/ml of serum from 18–20 snails, pooled from the two separate experiments. Standard deviations (SD) shown equal the logarithm$_{10}$ of the standard deviations.

Quantitative PFU assays were also carried out on the sera of 48 untampered snails (Group 9). No coliphages were detected.

DISCUSSION

There are several reports which suggest that invertebrates are capable of mounting an enhanced response to post-primary challenge(s). As examples, Evans *et al.* (1969) have reported that when the spiny lobster, *Panulirus argus,* is challenged with a Gram-negative bacillus identified as EMB-1, a hemolymph bactericidin is synthesized. More important, they reported that the bactericidin titer is increased as a result of secondary and tertiary challenge with EMB-1. More recently, Karp and Rheins (1980) have demonstrated a humoral response induced in the American

cockroach, *Periplaneta americana,* to the soluble protein complexes of honey bee (*Apis mellifera*) toxin and western cottonmouth moccasin (*Agkistrodon piscivorus*) venom. The response was specific for the original immunizing protein and a second challenge with the same antigen induced a classic secondary response in primed animals, thus suggesting the existence of immune memory. Also, Hildemann *et al.* (1977) have reported that there is development of a specific memory in the scleractinian coral *Montipora verrucosa* as a result of second-set grafts, and Hildemann *et al.* (1980a) have reported accelerated reactivity to second-set grafts in the sponge *Callyspongia diffusa.* Similarly, Langlet and Bierne (1977) have reported that there is enhanced rejection of second-set grafts among nemertines of the genus *Lineus.* The accelerated rejection of second- and third-set allografts has also been reported for the sea star *Dermasterias imbricata* (Karp and Hildemann, 1976).

Based upon the criterion of enhanced graft rejection, Cooper (1968, 1969, 1970) has promoted the idea that immunologic memory occurs among annelids; however, his results and interpretations have been challenged by Dales (1978) and Parry (1978).

Among molluscs, the enhanced clearance of T2 coliphage from the oyster *C. virginica* following second challenge (Acton and Evans, 1968) has been mentioned. Criticism of this study may be leveled at the fact that half-shell preparations were employed. Since bivalves, like gastropods, have an open circulatory system, the removal of one of the valves, which involves cutting the adductor muscle, could result in a nonphysiological state involving significant hemorrhage. Consequently, the results of that study remain open to question.

Lie and Heyneman (1975) and Lie *et al.* (1975) have reported what could be interpreted to be an enhanced reaction in a gastropod mollusc resulting from prior challenge. Specifically, they have found that when naturally resistant juvenile *B. glabrata* are challenged with normal miracidia of the digenean *Echinostoma lindoense* (Lie and Heyneman, 1975), or when susceptible snails are challenged with irradiated miracidia (Lie *et al.,* 1975), the parasites migrate to the heart where they become encapsulated by hemocytes and are destroyed. If such snails are subsequently challenged with normal miracidia, the parasites are destroyed rapidly by hemocytes near the penetration site in the head-foot. These observations suggest that initial challenge enhances the cidal effects of the hemocytic response to the second challenge with the parasite.

It now appears that *B. glabrata* is capable of recognizing certain viruses and eliminating them from their hemolymph. Specifically, these molluscs, as observed previously in crayfish (Sloan *et al.,* 1975) and blue crabs (McCumber and Clem, 1977; McCumber *et al.,* 1979), have naturally occurring levels of a soluble phage neutralization factor(s) in their serum, as shown by neutralization of T4D and T7 *in vitro.* Challenge of the snails with either type of phage prior to assaying the serum for the ability to neutralize phage does not induce an elevated level of this factor, and, in fact, snails previously challenged with T7 show decreased neutralization of T4D (Table I). Snails previously challenged with T4D have a higher titer than untampered snail serum when incubated with T4D *in vitro* but, due to the high standard deviation, this difference was not significantly different. These observations may indicate that the initial challenge of these snails with either T4D or T7 reduces the level of this factor in their hemolymph. It is noted that this naturally occurring factor appears at low levels in the serum since a 100-fold dilution of the serum prior to incubation with the bacteriophages reduces the neutralizing ability to the point that confluent plaques occur, as is observed when the phage are plated in the absence of serum.

Our finding of neutralization of T7 by *B. glabrata* serum is in contrast to that for the blue crab, where McCumber *et al.* (1979) observed that T3 and T7, two bacteriophages that are not cleared significantly *in vivo* (McCumber and Clem, 1977), are not neutralized *in vitro* whereas T2 phage is both cleared *in vivo* and neutralized *in vitro*. Furthermore, our finding of the ability of *B. glabrata* serum to neutralize T4D and T7 *in vitro* is in contrast to that for *C. virginica,* where Feng (1966) observed that serum from both noninjected oysters and oysters previously challenged with the bacteriophage *Staphylococcus aureus* phage 80 had no neutralizing effect on this bacteriophage *in vitro*. It is noted that Feng (1966) has reported that the clearance of *S. aureus* phage 80 is enhanced at higher ambient temperatures, *i.e.,* 15°C and 23.5°C as compared with 5°C.

The reduction in the mean serum titer of the bacteriophages T4D and T7 in the hemolymph of *B. glabrata,* therefore, appears to be due in part to a naturally occurring neutralization factor. However, since prior challenge of the snails with either bacteriophage does not stimulate higher levels of this factor, neutralization is probably not solely responsible for the more rapid reduction of phage titers *in vivo* in the serum of doubly injected snails. McCumber and Clem (1977), using radiolabeled viruses, have shown that T2, T4, and poliovirus are actively cleared from the circulation of the blue crab and not simply neutralized by the hemolymph. Although similar tracer studies have not been conducted in *B. glabrata,* the existence of an active, perhaps cell-mediated, clearance mechanism is the simplest explanation for our data. However, it is possible that some other neutralizing factor(s) are present in serum and are enhanced by previous challenge but are not demonstrable *in vitro, e.g.,* due to nonoptimal physiological conditions, the brevity of the assay, etc.

In conclusion, the data presented herein show that there is a reduction in the amount of infective bacteriophages in the hemolymph at 24, 48, and 72 h for T7 and at 24, 48, 72, and 96 h for T4D in doubly injected snails when compared to (1) snails that had been challenged with a single dose of coliphage and (2) snails that had been initially challenged with the Tris diluent or sham injected prior to challenge with bacteriophage. Thus, it would appear warranted to conclude that there is an enhanced ability in *B. glabrata* to clear and/or neutralize T4D and T7 *in vivo* that is inducible by previous homologous challenge. A comparison of *in vitro* and *in vivo* data implies that it is clearance rather than neutralization that is responsible for the enhancement effect. Whether this enhancement represents true "memory", *i.e.,* a separate, specific, and more rapid secondary response, or if it is simply a result of a prolonged primary response to the initial injection remains to be investigated.

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